Differential Phase Contrast in Hard X-ray Scanning Microscopy of Biological and Biomedical Samples

Stefan Vogt

Experimental Facilities Division - Advanced Photon Source
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Outline

- Why study trace metals in biology & life sciences?
- Why use hard X-ray fluorescence microscopy?
- Instrumentation
- Scientific applications:
  - As in plant cells
  - TiO$_2$-DNA nanocomposites in cells
  - Trace metals in plankton
  - Zn in heart muscle cells
- Differential Phase Contrast to visualize specimen structure
- Summary
Why Study trace elements / metals in biology and life sciences?

- Trace elements (metals) are fundamental, intrinsic components of biological Systems. Estimated: 1/3 of all known proteins contain metal cofactors as integral, catalytic components. These proteins often have regulatory or catalyzing functions, e.g.,
  - Cu binding chaperones (protein folding)
  - Zn in Zinc finger proteins: transcription factors in the cell nucleus
  - Fe in Haemoglobin; and necessary in Chlorophyll synthesis

- Metals can be linked to disease
  - Endogenous dysregulation, e.g., Alzheimer’s, ALS
  - Exogenous uptake, e.g., Pb, As, Hg

- Metals can be made use of in therapeutic drugs and diagnostic agents
  - Cis-platin in chemotherapy
  - Sb to treat Leishmaniasis
  - Gd in Magnetic resonance imaging (MRI)

➔ study distribution and quantity of these elements within cells to understand how they act

See e.g., Science 9 May 2003 (300 #5621) with Focus: “Metals Impacts on Health and Environment”
Why use x-ray-induced fluorescence to study trace metals?

- Simultaneously map 10+ elements
- No dyes necessary
- High signal/background ratio
  - sub-ppm (part-per-million) sensitivity, increasing with Z
  - quantitative
- Little radiation damage
- Large penetration depth (> 100 μm)
  - study whole cells, w/o sectioning
  - study ‘thick’ tissue sections
  - possibility to study hydrated “natural” samples using cryo
- Monochromatic incident beam: choose at which Z to stop excitation (e.g., excite As but not Pb)
- Straightforward to calculate fluorescence crosssection (quantification)
- Map chemical states by XANES

Detection Limit for Transition Elements: for 1 sec. acquisition time, 0.2 x 0.2 μm² spot, E=10 keV
### Comparison with other techniques:

<table>
<thead>
<tr>
<th></th>
<th>spatial resolution</th>
<th>object thickness</th>
<th>resolution limitation</th>
<th>Advantages/Disadvantages</th>
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<tbody>
<tr>
<td><strong>Light-microscopy</strong></td>
<td>200 nm</td>
<td>30 µm</td>
<td>wavelength</td>
<td>+ changes in living cells can be monitored</td>
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<td>- need dyes, competition w. proteins</td>
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<td>+/- typically see ions, and not total content</td>
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<td>- quantification difficult</td>
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<tr>
<td><strong>Hard X-ray-microscopy</strong></td>
<td>150 nm</td>
<td>10 – 100 µm</td>
<td>currently optics</td>
<td>+ no dyes (pot. close to native state)</td>
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<td>+ low background, high sensitivity</td>
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<td>+ simultaneously detect &gt;10 elements</td>
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<td>+ µ-XANES for chemical state mapping</td>
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<td>- long integration times</td>
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<td><strong>Electron-microscopy</strong></td>
<td>20 nm</td>
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<td>object thickness</td>
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<td>+ simultaneously detect &gt;10 elements</td>
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<td>- whole cells very difficult, sectioning necessary</td>
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Periodic table highlighting hard X-ray fluorescence

- **K-line Fluorescence typically used**
- **L-line Fluorescence typically used**

**Constituent of Earth's Crust**
- Intrinsic to Biological Systems
- Agents with Biological Activity

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**Intrinsic to Biological Systems**
- Agents with Biological Activity

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**Constituent of Earth's Crust**
- Elements important in biological systems.

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**Agent with Biological Activity**
- Elements essential for life processes.
2-ID-D/E X-ray microprobe beamline

Main branch 2-ID-D:
- $E = 5 - 30$ keV, $\delta = 150$ nm $\leftrightarrow 2 \cdot 10^9$ phot/s

Side branch 2-ID-E:
- $E = 7 - 17$ keV, $\delta = 250$ nm $\leftrightarrow 5 \cdot 10^8$ phot/s

- image sample by raster scanning the sample through focused beam
- acquire & analyse full XRF spectra at each scan position
- to quantify: compare with calibration curve

Detection of transmitted signal: absorption & diff. phase contrast

Schematic – NOT to scale
2-ID-E Hard X-ray Microprobe Facility

Epi-Fluorescence Microscope

Sample Holder

Sample in sample chamber, purge with He

Kinematic Mount
targeting cells

• select cells to scan using light microscopy
• record cell positions, do a coordinate system transf.
• in the XRM, these positions then give the location of the cells within <=5 microns

kinematic specimen mount

condenser

specimen

100x/0.9 NA micrograph

10 microns
Scientific applications
Elemental Distribution in Pteris cretica

Study intracellular As distribution in hyperaccumulating plant.

Marianna Kissel, Rich Reeder, et al
**TiO$_2$-DNA nanocomposites as intracellular probes**

- Cell is transfected with TiO$_2$-DNA nanocomposites (4.3 nm Ø)
- DNA is used to target nanocomposite to specific chromosomal region
- TiO$_2$ allows photocleavage of targeted DNA strand upon illumination
- Potential to be used for gene therapy

- Map Ti distribution using X-ray induced K$_{\alpha}$ fluorescence, to quantify the success rate of TiO$_2$-DNA transfection, and visualize target
- A: scan of a MCF7 cell transfected with nanocomposites targeted to nucleolus
- B: scan of a PC12 cell transfected with nanocomposites targeted to mitochondria

Trace metals in plankton and global carbon balance

- CO₂ sequestration in the ocean seems to be limited by the availability of Fe (necessary for Chlorophyll production).
- Standard approach to study Fe uptake: bulk analysis, BUT cannot distinguish between different organisms, or solid Fe particles not bio-available
- X-ray microscopy: separate and study individual organism

Light, epifluorescence and XRF images of diatom
- Si maps onto diatom frustule
- Fe is concentrated in/near the chloroplasts (epi)

Signaling Cardiac Muscle Injury with Zn ???

B. Palmer, S. Vogt, Z. Chen, R. Lachapelle
What properties of cardiac hypertrophy are compensatory? How can hypertrophy be reverted?
Cardiac hypertrophy due to bigger cardiac myocytes (heart muscle cells)

Normal Size
120 um x 25 um x 10 um

Hypertrophic Compensation for High Blood Pressure:
120 um x 30 um x 12 um

What other properties accompany compensation?
Low atomic-number elements in a myocyte cause action-potential and trigger force.

- Potassium (K)
- Calcium (Ca)

High atomic-number elements in a myocyte.

- Iron (Fe)
- Copper (Cu)
- Zinc (Zn)
What are these patterns of Fe, Cu, and Zn?

Fe and Cu cosegregate opposite Zn.
Is Muscle LIM Protein (MLP) responsible for striations of Zn?

MLP is also implicated as stress sensor. MLP released by injury is thought to activate GATA-4 and gene expression.
But:

*How to correlate element distribution with biological structure?*
How to correlate element distribution with biological structure?

Hard X-ray microscopy: great sensitivity for medium/high Z elements,

but mapping of biological mass and structure (mostly C,N,H) difficult:
    very low photoelectric absorption
    very low fluorescence yield

at the same time:

• exact correlation of elemental maps with biological structure critical!!

Are these the same striations???

• as well as find cells quickly!
Why use phase information?  \[ n = 1 - \delta - i\beta \]

- detect phase contrast in addition to absorption contrast
  - for fast scans for exact cell / tissue targeting
  - to acquire structural, C-based information from the specimen

\[ \Rightarrow \text{use phase shift (delta)} \]
Schematic DPC

- differential detection in direction of phase gradient

DPC signal = detector_1 – detector_2
Implementation of unidirectional DPC

- differential detection using two ion chambers and a motorized knife edge
- DPC signal = left-right/sum=(DS_IC – 2*DPC_IC)/DS = 1- 2*DPC_IC/DS_IC

In practice: DPC_IC / DS_IC (can do hardware calculation, and detect direct DPC signal)
absorption and differential phase images of mammalian cells using ion chambers

**step** scan w/ stepping motors:
50x50 microns,
0.4x0.4 microns steps
detected flux: $\sim 4 \times 10^9$ ph/s
duration: $\sim 4$ h

**fly** scan w/ stepping motors:
50x50 microns,
0.2x0.2 microns steps

detected flux: $\sim 4 \times 10^9$ ph/s
duration: $\sim 4$ h

Even with standard detectors, fly scans can enable detailed pre-view scans
Example: cardiac myocyte (heart muscle cell)

Absorption:

step scan: 45x28 microns, 0.25x0.25 microns steps, 1 second dwell time per pixel, 10 keV incident Energy.
S Cl Zn

Absorption:

VLM

DPC:

step scan: 45x28 microns, 0.25x0.25 microns steps, 1 second dwell time per pixel, 10 keV incident Energy.
Enter the segmented detector:
DPC w/ segmented detector

incident X-rays

upstream pinhole

upstream ion chamber (US IC)

zone plate

order sorting aperture (OSA)

TFY

sample

EDS

Segmented Detector
**DPC w/ segmented detector**

Horizontal: \( DPC_0 = (dl+ul) - (ur+dr) \)
\( DPC_{45} = ul - dr \)

Vertical: \( DPC_{90} = (ul+ur) - (dl+dr) \)
\( DPC_{135} = ur - dl \)

**NOTE:** setup not yet optimized for hard X-rays (e.g., 10 keV)

- use Al-filter to reduce detected flux (attenuate by 98% !!!)
- detector triggered every 500us
- read detector output using Nova V-to-F, into multi-channel-scaler with external channel advance
- focal spot typically NOT round but elliptical (mismtach to detector chip)
Cardiac myocyte (heart muscle cell):

Raw signal from segmented Detector

scan: 50x70 microns, 50x100 nm steps, detected flux: $\sim 10^8$ ph/s, 1 ms dwell

center top : ct  center middle : cm  center down : cb  down-left : dl

top-up

up-left : ul  up-right : ur  down-left : dl  outer ring : oo
DPC from segmented Detector

Segmented Detector

scan: 50x70 microns, 50x100 nm steps,

Z-bands
Repeat with only 0.1 ms dwell time …

'raw' signal

'glitch' due to switch from base speed to backlash speed

Slower movement w/ backlash speed

acceleration

deceleration

signal/dwell time

~ 10^4 photons/pixel

Top-up

First pixels: overflow

fly scan w/ stepping motors: 50x70 microns, 0.1 ms dwell; 1000x700 pixel; scan time: ~30 min. Estimated scantime w/o fly scans: 20h
DPC of cardiac myocytes with dedicated detector

0.1 ms dwell
total scan time: ~30 min

1 ms dwell
total scan time: ~1 h

remaining overhead: flyback & y motion
Cardiac myocyte at 2.5 ms dwell, 0.2 micron steps, 80x80 micron steps
Cardiac myocyte at 10 ms dwell, 0.2 micron steps, 80x80 micron steps

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</table>
Example: 300x300 μm² area of brain section (Collaboration w. E. Bigio et al)
Example: 300x300 μm² area of brain section

visible light microscope

Overview scan: X-ray absorption contrast
Example: 300x300 μm² area of brain section

visible light microscope

Overview scan: X-ray diff. phase contrast

Example: 300x300 μm² area of brain section
Summary: Hard x-ray fluorescence microscopy for biological systems

- Focus hard x-rays (e.g., 10keV) on sample, raster scan sample through focal spot, collect characteristic x-ray fluorescence at each position to determine elemental content
- Current spatial resolution: 150 nm
- X-ray induced x-ray fluorescence
  - High trace element sensitivity \(10^{-17} \text{g}\) for medium to high Z elements
  - Quantification to ppm level for most metals
  - Combine with micro-XANES to determine speciation
Summary Differential Phase Contrast:

- Differential Phase Contrast improves contrast for biological mass (‘C’) significantly!
- DPC data ‘for free’ during acquisition of step scans
- Improves correlation of light micrographs with elemental maps (e.g., exact position of Z-bands with respect to maps)
- Fly-scans give high-res images & improve pre-positioning of scans

Ionchamber based DPC:
- Advantages:
  - Simple & Cost-effective
  - Fast switching to view of transmitted X-rays
- Disadvantages:
  - Only sensitive to phase gradient in one direction (with some compromise in 2)
  - Sensitivity limit scan speed to > 1ms for overview scans, and to >10 ms for scans to correlate structure with elemental content

Segmented Detector:
- Photon statistics easily allows dwell times of <0.1 ms
- Extraction of all DPC signals possible w/o use of CCD camera
Next step

- Biologists are interested in elemental concentration, ideally in water (not just total quantity, as we measure).
- If that is not possible, quantitation in mmol/kg dry weight is the next best thing.
- Develop algorithm to ‘integrate’ back to the protein thickness (and dry weight) from the DPC signal
Instrumentation Challenges

- Speed (photon statistics allow <= 10 microseconds dwell time)

and

- Sensitivity
  - Collect (weak) small angle scattering in the outermost segment, with strong direct signal in the inner 7.
An example where DPC currently does not work …
Angiogenesis (growth of new blood vessels)

- controlled through growth factors (on) and inhibitors (off)
- Disease states:
  - Insufficient angiogenesis, e.g., stroke
  - Excessive angiogenesis, e.g., cancer
    - feeds tumor
    - provides ‘transport’: metastasis
- currently ~65 antiangiogenic drugs in clinical trial for cancer treatment

http://press2.nci.nih.gov/sciencebehind/
An in vitro angiogenesis assay

Human Microvascular Endothelial Cells (HMVEC)
Sensitivity of angiogenesis to Cu/Zn chelation
Cu in Angiogenesis

- Blood vessel formation is sensitive to Cu chelation
- Control of angiogenesis could be critical tool to combat cancer

$t = 0.5h$

$t = 1h$

$t = 2h$
What is happening at the tip of tube growth cones?

- Secretion of a cupric metalloprotease?
- SPARC (secreted protein, acidic and rich in cysteine), matricellular protein?
- Matrix remodeling?

Future experiments:
- Verify *in vivo*: compare Tumor tissue (lots of angiogenesis) with Normal Tissue
- Compare with neuronal growth processes which are quite similar